

2 1. Supplemental Figures and Supplemental Figure Legends

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```
syntaxin-1A -----MKDRTEQLRTAKSDSDDDDDVAVTVD-----RDRFMDEFFE 35
syntaxin-2 -----MRDRLPDLTACRND-----DGDTVVVVE-----KDFMDDFFH 34
syntaxin-3 -----MKDRLEQLKAKQLTQDDDTDAVEIA-----IDNTAFMDEFFS 37
syntaxin-4 -----MRDRTELRQGGDSSDEEDKERVAVLV-----HPGTARLGSPDIEFFH 43
syntaxin-5 -----MIPRKRYSKNTDQGVYLGSLKTVLSPATAGSSSSDIAFLPPPVTLVP 49
syntaxin-7 -----MSY-----TPGVGGDPAQ 13
syntaxin-11 -----MKDRLAELLDLKQYDQFPDGDDEFDSPHEDIVF-----ETDHILESLYR 46
syntaxin-T -----MSYGPLDMYRNP GP-----SGPOLRDFSS 24
syntaxin-16 MATRRLTDAFLLLRNNSIQNRQLLAEQVSSHITSSPLHSRSIAAELDELADDRMALVSGISLDPEAAIGVTKRPPPKWVD 80
syntaxin-17 -----MSEDEEKVKLRR-----LEPAIQKFIK 22
syntaxin-18 -----MAVDITLLFRASVTKVTRNKALGVAVGGGVDGSRDELFR-----RSPRPKGFDFSS 51

syntaxin-1A QVEEIRGFIDKIAENVEEVKRRKHSAILASPNPDEKTK--ELEEELMSDIKKTANKVRSKLSKISIEQ----- 98
syntaxin-2 QVEEIRNSIDKIQYVEEVKKNHSIILSAPNPEGKIK--ELEDLNKEIKKTANKIRAKLKAIEQS----- 98
syntaxin-3 EIEETRLNIDKISEHVEEAKKLYSIIISAPIPEPKTK--DDLEQLTTEIKKRANVNRNKLKSMEKH----- 101
syntaxin-4 KVRTIROTIVKLGKNGVQELEKQOVTIILATPLPEESMK--QELONLRDEIKQLGREIRLOKKAIE----- 105
syntaxin-5 PPPDTMSCRDRITQEFLSACKSLQTRONGIQTNKPAIRAVRQRSEFTLMAKRIGKDLNSNTFAKLEKLTILAKRKSIFDDKA 129
syntaxin-7 LAQRISSTOKITQCSVEIQRT-LNQLGTPOQDSPELR--QQLQKQOYTNQLAKETDKYIKKEFGSL----- 76
syntaxin-11 DIRDIQDENQLLVADVKKRLGKQNAFLTSMRRLSSIK--RDTNSIAKAIKARGEVIHCKLRAMKEL----- 110
syntaxin-T IIDTCSGNVQRIISQATAQIKNL-MSQLGTQKODSSKLO--ENLQLOHSTNQLAKETNELIKELGSL----- 87
syntaxin-16 GVDEIQYDVGRIRKQMKMELASLHDKHLNRPTLDDSSSEEAHAEITTOEITQLFHRQRAVQALPSR----- 146
syntaxin-17 IV--IPTDLRLRKHQINIEKYQRCRIWKKLHEEHINAGRTVQQLRSNIRIEIKLCLKVRKDDLVL----- 86
syntaxin-18 RAREVISHIGKLRDFLEHRKDYINAYSHTMSEYGRMTDTERDQIDQDAQIFMRTCSEATQQLRTE----- 117

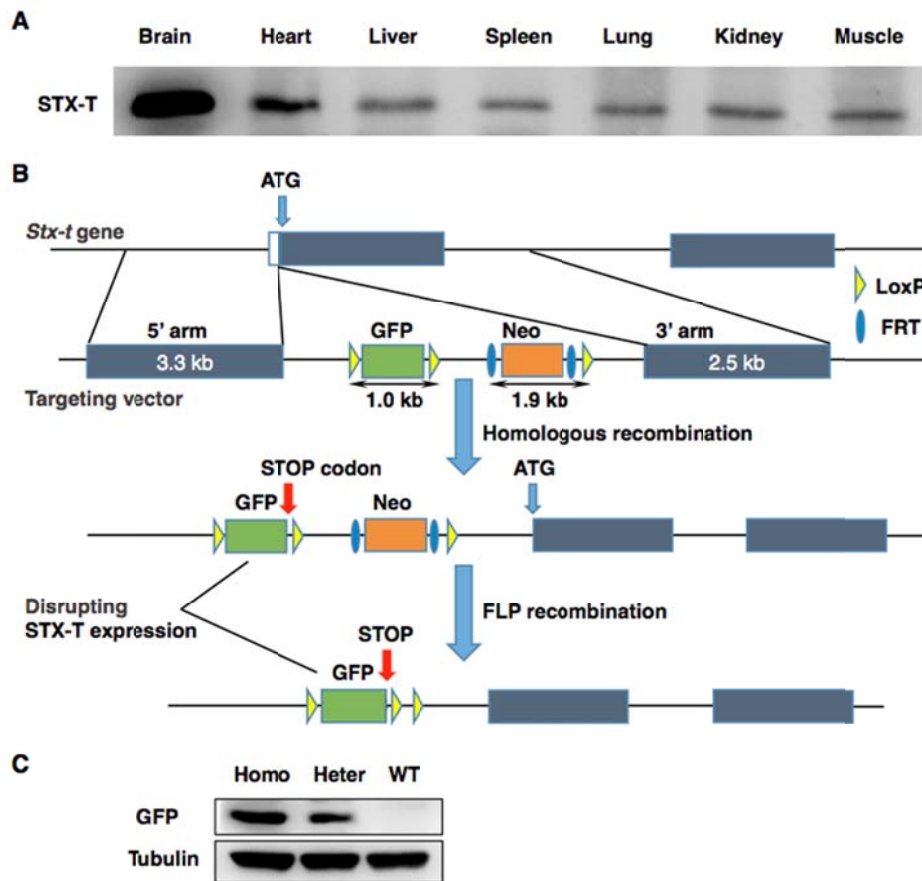
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syntaxin-2 -----FD-QDESGNRTSVDLRIRRTQHSVLSRKFVEAMAENEAQTLFRERSKG---RIQR--- 150
syntaxin-3 -----IEEDEVRSADLRIRKSQHSVLSRKFVEVMTKYNEAQQVDFRERSKG---RIQR--- 151
syntaxin-4 -----PQEEADENYSVNTMRMKTQHGVLVQQFVELINKCNMSQSEYRERKNVE---RIRR--- 158
syntaxin-5 VEIEELTYIIKQDINSLNKQIAQLQDFVRAKGSQSRHLOTHSNTIIVVLSQSKLASMSNDFKSVLVRVTENLKQQRSS--- 206
syntaxin-7 -----P-----TTPSEORQRKIQKDRVAEFTTSLTNFQKQVQRAAEREKEFVAARVRA--- 124
syntaxin-11 -----SEAAEAQHGPSAVARISRAQYNALTLTFORAMHDYNAQEMKQRDNCKI---RIQR--- 163
syntaxin-T -----PL-P-----LSTSEORQORLOKERLMDNFSAAALNNFOAVORRVSKEKESIAARARA--- 137
syntaxin-16 -----AR-A-----CSEQEGRLIGNVVASAQAALQELSTSPRHAQSGYLKRMKN---REIR--- 193
syntaxin-17 -----LKRMDIPVKEEASAATAEFLQLHLSEVEELKKQFNDEETLLQPLTR--- 133
syntaxin-18 -----AHKE-----IHSQQVVEHRTAVLDFIEDYLRKRVCKLYSEQRAIRVRRVDDKRLSKLEP 171

syntaxin-1A -----QLEITGR-TTTSEELEDMLESGNPAIFAS-----GIIMDSI-----SKQALSE---IE 196
syntaxin-2 -----QLEITGR-TTTDDELEEMLESCKPSIFTS-----DIISDSQI-----TRQALNE---IE 195
syntaxin-3 -----QLEITGK-KTTDEELEEMLESGNPAIFT-----SGIIDSQI-----SKQALSE---IE 195
syntaxin-4 -----QLKITNAGMVSDEELEQMLDSGQSEVFS-----NILKDTQV-----TRQALNE---IS 204
syntaxin-5 -----RREQFSRAPVSAALPLAFLNHLGGGAVVLGA-----ESHASKDVAIDMMSDRTSQQLLIDEQQSYIQ 267
syntaxin-7 -----SSRVSGSFPEDSSKERNLVSWESQTOPOV-----QVQDBEI-----TEDDLRL---IH 169
syntaxin-11 -----QLEIMGK-EVSGDQIEDMFEQKWDVFS-----NLLADYVK-----ARALNE---IE 208
syntaxin-T -----GSRLSAEERQREEQLVFSDFSHHEWQMS-----Q---BDEVAI-----TEQDLEL---IK 182
syntaxin-16 -----SQHFFDT-----SVFLMDDGDDNTLYH-----RGFTBDQDLV-----VEQNTLM---VE 234
syntaxin-17 -----SMTVGGAFHTTEAEASSQSLT-----QIYALPEIP-----QQQNAE----- 170
syntaxin-18 EPNTKTRESTSSEKVSQSPSKDSEENPATEERPEKILAETQPELGTWGDGKGEDELSPPEIQMFEQENQRLIGE---MN 247

syntaxin-1A TRHSEIKLENSIRELHDMFMMDMMLVESQGMIDRIEYVNEHVDYVERVSDTKKAVKYQSKARRKKIMIIICCVILG 276
syntaxin-2 SRHKDMKLETSIRELHEMFMDMAMFVETQGMINNIERNVMNATDYVEHAKKEETKKAIKYQSKARRKKWIIIVAVSVVL 274
syntaxin-3 GRHKDVRLESSIRELHDMFMDIAMLVENQGMELDNIELNVMHTVDHVEKARDETCKKAVKYQSKARRKLIIVLVLVVL 274
syntaxin-4 ARHSEIQLERSIRELHDIFFLAFVEVMEQGMINRIEKNILSSADYVERQOEHVKTALENQKARKKKVLIIVAVSIT 283
syntaxin-5 SRADTMQNIESTIVELGSIQQLAHMVKEQEBTIQRIIDENVLGAQLDWEAAHSEI---LKYFQSVTSNRWLMVKIFLILI 344
syntaxin-7 ERESSRQLEADIMDINEIFKDLGMMIHEQGDVIDSIKANVENNEVHVQANQQLSRAADYQRKSRKTLICIFILVIGV 249
syntaxin-11 SRHRELRLESRIRDVHELFLQMAVLVEKQADFLNVIELNVQKTVDYTGQAKAQRKAVQYEEKNPCRT---LCCFC 283
syntaxin-T ERETRRQLEADIDVNOIFKDLAMMIHQDQEDTDSIRANVESSEVHVERATEQLQRAAYYQKSRKKMCIIVLVLSVII 262
syntaxin-16 ERETRRQIVQSIIDLNEIFRDLGAMIVEQGTVLDRIIDYVNEQSCIKTEDGLKQLHKAQYQKKNR-KMLVIL-ILFVII 312
syntaxin-17 ---SNETLEADLHLSQLVTDVFLVNSQGMKIDSIADHVNSAVNVEETKNLGGKAAKYLALPVAGALIGGMVGGP 246
syntaxin-18 SLFDETRQLEGRVVEISRLQEIFTEKVLQOEAELSDIHLQVVGATENAKENEDIREAIKNNAGFR--VMILF-FLVMCS 324

syntaxin-1A IVIA--STVGGIFA----- 288
syntaxin-2 VAI--ALIIIGLSVGN----- 288
syntaxin-3 LGL--ALIIIGLSVGN----- 289
syntaxin-4 VVGL--AVITGVTVVG----- 297
syntaxin-5 VFFI--IFVVFILA----- 355
syntaxin-7 AINS--LIINGLNE----- 261
syntaxin-11 PCIK----- 287
syntaxin-T LIG--LIITWLVYKTK----- 276
syntaxin-16 IVI--VVLVGVKSR----- 325
syntaxin-17 IGLLAGFKVAGIAAALGGGVLGFTGGKLIQRKKQKMMKELTSSCPDLPQSQTKKCS 302
syntaxin-18 FSL--FLDNYDS----- 335
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3 **Supplementary Figure 1. Multiple alignment of 11 Qa-SNAREs.** Syntaxin-T stands
4 for syntaxin 12/13. Amino acids with more than 80% similarity are highlighted. The
5 backgrounds of similar amino acids are dark gray, while the backgrounds of identical
6 amino acids are light red. A red star indicates the conserved glutamine residues in helix
7 bundle region for fusion complex forming. The C-terminal transmembrane domains are
8 underlined. GenBank accession numbers for the human Qa-SNARE family are as
9 follows: syntaxin-1A , NP_004594.1; syntaxin-2, NP_919337.1; syntaxin-3,
10 NP_004168.1; syntaxin-4, NP_004595.2; syntaxin-5, NP_003155.2; syntaxin-7,
11 NP_003560.2; syntaxin-11, NP_003755.2; syntaxin-T, NP_803173.1; syntaxin-16,
12 NP_001001433.1; syntaxin-17, NP_060389.2; syntaxin-18, NP_058626.1.



14

23 **Supplementary Figure 2. Generation of *Stx-t* knockout mouse and STX-T expression**

24 **level in tissues.** (A), Immunoblot analysis of STX-T expression in E18.5 mouse tissues.

25 Protein samples prepared from E18.5 mice are blotted with antibodies against STX-T.

26 STX-T expresses ubiquitously and highly in brain and heart. (B), Schematic of generating

27 *Stx-t* knockout mouse. To disrupt STX-T expression, the GFP gene with stop codon is

28 inserted at the 10 bp upstream side of ATG start codon. Targeting vector is used for

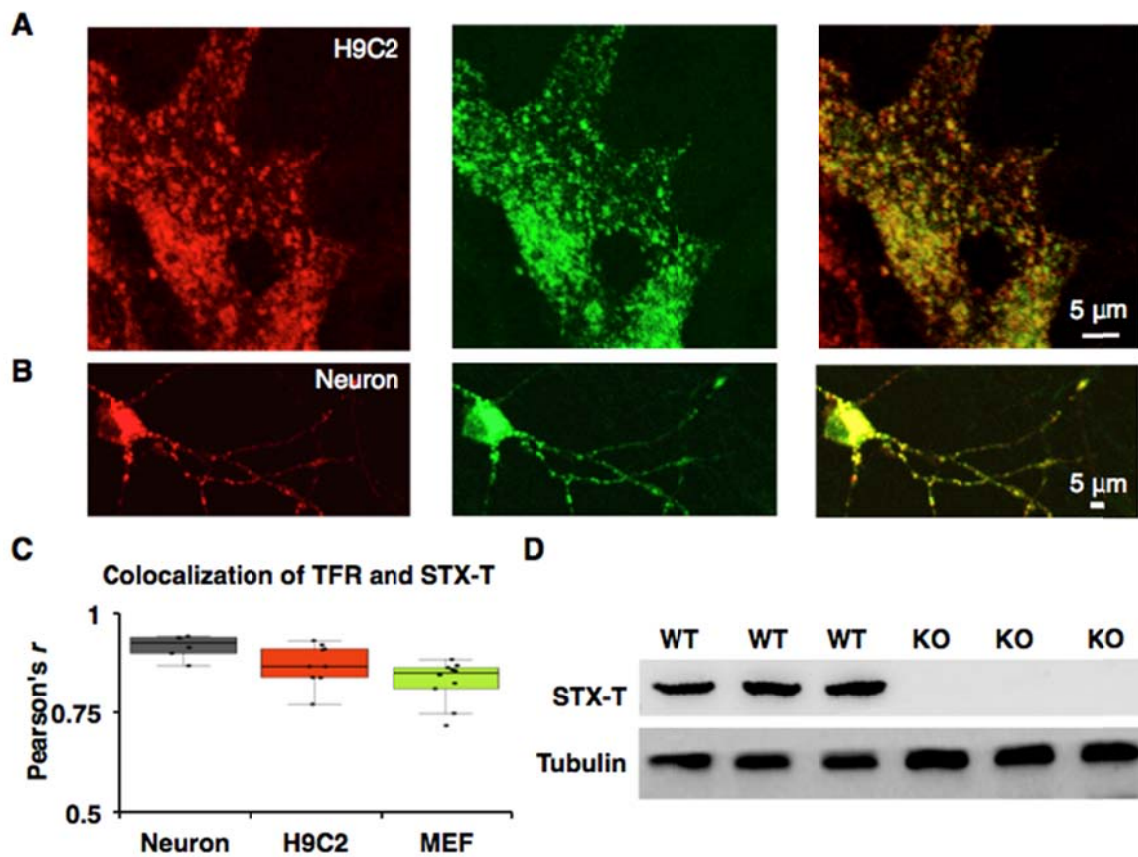
29 homologous recombination in embryonic stem cells to generate *Stx-t* knockout mice,

30 which are further intercrossed with FLP mice to remove Neo gene. Neo, Neomycin

31 resistance gene; FRT, flippase recognition target; FLP, flippase; LoxP, locus of X-over

26 P1.(C), Immunoblot analysis of GFP. Brain homogenates are prepared from P0 wildtype
 27 (WT), heterozygous (Heter) and homozygous (Homo) mice, and sequentially blotted with
 28 antibodies against GFP (top panel) and loading control tubulin (bottom panel).

27

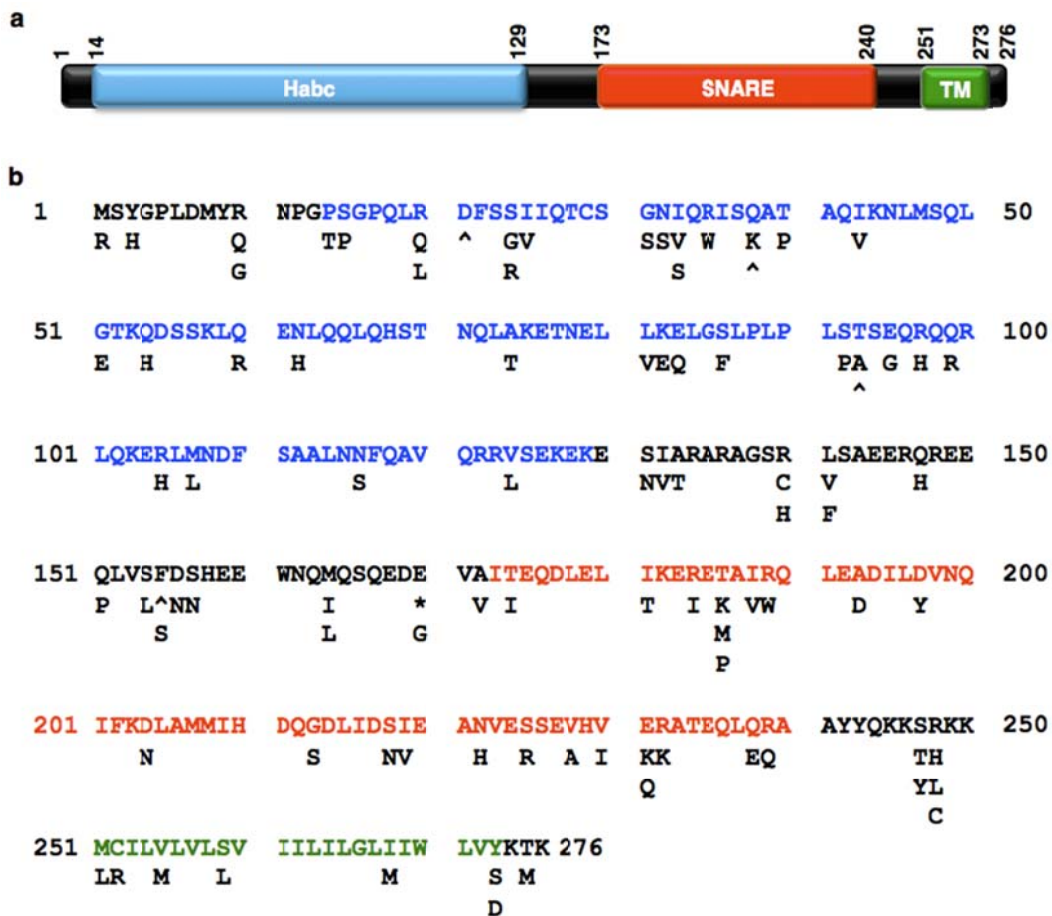


28

29 **Supplementary Figure 3. Colocalization of STX-T (red, RFP tagged) with TFR.**

32 (A)-(C), Colocalization of STX-T (red, RFP tagged) with TFR (green, GFP tagged) in
 33 H9C2 (2-1) cell line (A) and primary hippocampal neuron (B) respectively. The scale
 34 bars represent 5 μ m. (C), Scatterplots with boxplots show correlation coefficient (mean \pm

37 SD) of STX-T and TFR colocalization in neuron (0.92 ± 0.03 , $n = 6$), H9C2 (0.87 ± 0.05 ,
 38 $n = 9$) and MEF (0.83 ± 0.05 , $n = 10$). (D), Immunoblot analysis of STX-T expressions in
 39 Mouse embryonic fibroblasts (MEFs) of wildtypes (WT) and homozygotes (Homo).
 40 Triplicates of WT and KO MEF samples are blotted with antibodies against STX-T (top)
 41 and loading control tubulin (bottom).



38

41 **Supplementary Figure 4. Known genetic variations within the coding region of**
 42 **human *Stx-t* gene.**(A), Human STX-T possesses a single C-terminal transmembrane
 43 domain (TM, green), a SNARE domain (SNARE, red), and an N-terminal regulatory

41 domain (Habc, blue). (B), The non-synonymous cSNPs and small-scale variations of
42 STX-T. A total of 90 non-synonymous cSNPs of STX-T are shown under reference
43 amino-acid residues, and synonymous cSNPs of STX-T are not shown. The star (*)
44 symbol represents stop-gained variant, and caret (^) marks represent frameshit variants.
45

46 **2. Materials and methods**

47 **Generation of *Stx-t* knockout mice**

48 The *Stx-t* disruption vector contains a gene sequence *loxp-GFP-PolyA-loxp* which was
49 inserted into the site between downstream of *Stx-t* promotor and the 10 bp upstream of
50 start codon. The sequence of *GFP* has a stop codon and neomycin resistance gene
51 followed by *frt* site. The targeting construct was electroporated into embryonic stem (ES)
52 cells to replace the targeted genome sequence by homologous recombination. ES cells
53 transfected after 24 hours were cultured with the medium containing 300 mg/L of G418
54 and 2 μ M of Ganciclovir. ES colonies, resistant to G418 and Ganciclovir, were picked,
55 cultured, analyzed for correct integration and confirmed by PCR analysis and sequencing.
56 Chimeras were generated from ES clones by blastocyte injection, backcrossed with
57 C57BL/6J, and heterozygous *GFP* knockin offsprings were confirmed by PCR analysis
58 and sequencing. Heterozygous mice containing the *GFP* knockin sequence were
59 intercrossed to generate *Stx-t* knockout mice. The day detecting vaginal plug is
60 designated as embryonic day 0.5 (E0.5) and all born and alive mice to be dissected have
61 been anesthetized by isoflurane. All experiments were conducted in accordance with the
62 guidelines of the Institutional Animal Care and Use Committee of the Institute for
63 Nutritional Sciences, Shanghai Institute for Biological Sciences, Chinese Academy of
64 Sciences.

65

66 **Isolation of Mouse Embryonic Fibroblasts (MEFs)**

67 Sacrifice anesthetized pregnant mouse at embryonic 17.5 and dissect out the uterine,
68 immersing into 75%(v/v) ethanol and rinsing with PBS without Ca^{2+} and Mg^{2+} (Gibco,
69 Invitrogen). Separate each embryo from its placenta, remove head, limbs and viscera and
70 mince the remaining tissue into pieces as small as possible. Use part of tail for genotype
71 identification. Digest the tissues in fresh 15 ml 0.05% trypsin for 30 min at 37 °C, and
72 shake the 50 ml tube every 10 min. Inactivate the trypsin by adding same volume of MEF
73 medium (10 ml FBS plus 90 ml DMEM), centrifuge cell at 1000 g for 5 min and then
74 remove the supernatant, repeat once time; Add 10 ml of MEF medium and transfer to a
75 55 cm² Petri dish for incubation.

76

77 **The internalization and recycling of transferrin in MEFs**

78 After incubating with 25 µg/ml conjugated with Alexa Fluor-546 for 30 min or 60 min,
79 MEF cells were live chased with 100 µg/ml non-fluorescent holo-TF (Calbiochem, Cat
80 No: 616420), the living imaging were recorded for about 10 min with the customized
81 fluorescence microscope with Evolve-512 EMCCD (Photometrics Ltd., USA) as camera
82 and Optoscan monochromator (Cairn Research Ltd., UK) as light source, and images
83 were taken every 5 seconds. The images were analyzed with ImageJ plugins “Intensity v
84 Time monitor of Stack-T function” and the fluorescence intensity was normalized with
85 the mean value of first 15 images before adding holo-TF.

86

87 **Statistical analysis**

88 Data are pooled from or repeated for each condition at least three independent
89 experiments. No statistical methods were used to predetermine sample size. Data of live
90 chasing in Fig. 2B are represented with mean values and the standard error of mean
91 (SEM) (Mean \pm SEM), All data in text or figures are indicated with mean values and the
92 corresponding standard deviation (SD) (Mean \pm SD). The genotypic distribution of
93 heterozygous offspring is analyzed using Chi-squared test (χ^2), and other results were
94 analyzed statistically by two tailed Student *t-test*. When the value *P* is less than 0.05,
95 differences are considered significant.

96

97 **Westernblot**

98 Mouse tissues were homogenized in cold lysis buffer (1% Triton X-100 and 1%
99 DOC in Tris-buffered saline buffer) with phosphatase and protease inhibitors using micro
100 tissue grinders (Kimble 749540-0000, USA), incubated on the ice for 30 min to lysis cell
101 completely, while MEF cells could directly be grinded, centrifuged at 12,000 rpm for 15
102 min at 4 °C, the supernatants (protein lysates) were collected and its concentration were
103 determined by BCA protein assay kit (CW BIO, Cat NO: CW0014). Extracted proteins
104 were separated using 4~20% gradient gel electrophoresis and electrotransferred to
105 Protran Nitrocellulose membranes (PerkinElmer) with a constant voltage of 120 for 1.5
106 hours. The transferred membranes were blocked by 5% nonfat dry milk in Tris-buffered
107 saline with 0.05% Tween 20 for 1 hour at room temperature, incubated with first
108 antibody for overnight at 4 °C with gentle shaking, washed in Tris-buffered saline with

109 0.05% Tween three times (10 minutes per time), finally incubated for 1 hour with anti-
110 rabbit (GE, 1:2000, Cat No: NA934) or anti-mouse (Cell Signaling, 1:2000, Cat No:
111 7076S) second antibody at room temperature and washed three times again in Tris-
112 buffered saline with 0.05% Tween. First antibodies were used recognizing Syntaxin12/13
113 (Abcam, 1:1000, Cat No: ab13261), β -Tubulin (Sigma, 1:2000, Cat No: T4026), GFP
114 (Abmart, 1:2000, Cat No: P30010), α -Actin (Abmart, Cat No: M20010) The
115 immunoblots were visualized with ECL Western Blotting Substrate (Pierce, Cat No:
116 32109) and exposed the blots to imager (GE, ImageQuant LAS 4000 mini).

117

118 **Plasmid construction, cell culture and transient transfection**

119 The DNA fragments encoding TFR and STX-T were amplified from rat brain
120 genomic complementary DNA (cDNA) and inserted into the expressing vector pEGFP-
121 N1 Vector (Invitrogen) and TagRFP-T-N1 vector respectively. Primary hippocampal
122 neuron culture process was described previously(Kang et al., 2008). H9C2 (2-1) cell lines
123 obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) was
124 cultured in DMEM with 10%FBS at 37 °C in 95% air and 5% CO₂. Cells were seeded at
125 a density of 1×10^4 cells per cm² and then transfected transiently by calcium-phosphate
126 method. TFR-GFP construct was co-transfected with STX12-RFP vector. After
127 transfection, cells were cultured for additional two days and imaged with confocal
128 microscope (FV1000; Olympus), the colocalization between TFR-GFP and STX12-RFP
129 was analyzed with imageJ plugin “colocalization Indices” (by Kouichi Nakamura, Kyoto
130 University) and represented with correlation coefficient.

131

132 **Whole blood collection and analysis**

133 Blood smears were made by putting a small drop of blood on a glass slide and then
134 spreading in a thin film over the slide using the edge of another slide. Air-dry blood
135 smears were stained with Wright-Giemsa stain (Solarbio, Cat No: 1020) according to the
136 manufacturer's manual. A volume of 20 μ l whole bloods were collected by using spray-
137 coated EDTA•K₂ collection tubes from decapitated E18.5 embryos, diluted to final
138 volume 200 μ l, and assayed in XT 2000i Automated Hematology Analyzer (Sysmex).

139

140 **Immunostaining**

141 MEF cells seeded on glass coverslips were starved for 2 hours in DMEM medium
142 without serum, placed on ice for 10 min and then incubated with 25 μ g/ml Transferrin
143 (TF) conjugated with Alexa Fluor 546 (Invitrogen, Cat No: T23364), being washed one
144 time with PBS and fixation for 30 min at room temperature (RT) in 4% FSB solution
145 containing 4% formaldehyde (Polyscience Inc.), 4% sucrose (Sigma-Aldrich) in 1x PBS,
146 cell were rinsed three times with 1x PBS, permeabilized and blocked with 0.5% Triton X-
147 100 and 5% serum in 1X PBS for 30 min at RT, incubated with early endosome antigen 1
148 (EEA1) antibodies (BD, 1:200, Cat No: 610456) over night at 4 °C, rinsed three times
149 with 1x PBS, incubated with Alexa 488 F(AB') goat anti-mouse secondary antibody
150 (Invitrogen, 1:400, Cat No: A-10684) for 30 min at RT, imaged with confocal
151 microscope and also analyzed the colocalization rate between TF-Alex 546 and EEA1.

152

153 **SNP analysis**

154 Known single nucleotide polymorphisms (SNPs) and small-scale variations of *Stx-t*
155 gene were retrieved from the dbSNP database (NCBI, build 148).